Relationship Between Glycemic Control and Collagen-Linked Advanced Glycosylation End Products in Type I Diabetes

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OBJECTIVE — To evaluate the relationship between glycemic control over a 3-yr period and tissue levels of advanced glycosylation end products. The development of renal failure, blindness, and generalized vascular occlusion continue to be the most serious ravages of diabetes. Tissue glycosylation and AGEs are felt to play an important role in the development of these sequelae, but no data are available on the relationship between AGEs and long-term glycemic control.

RESEARCH DESIGN AND METHODS — We studied 48 subjects with type I diabetes. Glycemic control was determined by mean levels of HbA_1, and AGEs were determined on collagenase digests of skin collagen by fluorescence at excitation/emission readings of 335/385 and 370/440 nm.

RESULTS — To evaluate the relationship between glycemic control and AGE levels, control was classified as good (≤8.5%), fair (>8.5% but ≤10%), or poor (>10%) on the basis of mean HbA_1 levels during 1- and 3-yr periods. Analysis of the mean AGE levels for each level of glycemic control over 1–3 yr showed that AGEs differed significantly across categories of glycemic control ($P = 0.04$ and 0.003), with the lowest AGE levels associated with good and the highest with poor glycemic control. The relationship also was highly significant when adjusted for age, sex, and duration of diabetes, and when examined by Pearson's correlation coefficients ($P = 0.02$ and 0.008).

CONCLUSIONS — Finding a relationship between glycemic control over 1–3 yr and tissue levels of AGEs suggests that AGEs can be modified and possibly reversed by improved glucose levels.

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TYPE I DIABETES, INSULIN-DEPENDENT DIABETES MELLITUS; AGE, ADVANCED GLYCOSYLATION END PRODUCT; RIA, RADIOIMMUNOASSAY; FU, FLUORESCENT UNIT; CV, COEFFICIENT OF VARIATION; ANOVA, ANALYSIS OF VARIANCE; ANCOVA, ANALYSIS OF COVARIANCE.

The development of renal failure, blindness, and generalized vascular occlusion continue to be the most serious ravages of diabetes, particularly in those with type I diabetes. Nonenzymatic glycosylation and the formation of AGEs on vascular and other tissues is felt to play an important role in the development of these long-term vascular sequelae associated with diabetes (1). Diabetes-related hyperglycemia (or elevated levels of other hexoses, pentoses, or their phosphorylated derivatives) can result in the increased formation of Amadori products, which act as precursors for the production of AGEs (2–5). The presence of these AGEs on vascular structures is felt to be of particular importance in the pathogenesis of diabetic microvascular and macrovascular sequelae. The accumulation of these products on collagens and other proteins can produce functional and structural changes in vascular tissues by multiple mechanisms (1), and several studies have shown that tissue levels of AGEs are increased in association with diabetic nephropathy and retinopathy (6,7).

Little information exists on the relationship between glycemic control and tissue levels of AGEs. One short-term study has shown no change in tissue levels of AGEs after 4 mo of improved glycemic control, but longer term studies have not been performed (8). Although Amadori products are slowly reversible and reflect ambient glucose concentrations (9), AGEs are chemically irreversible (10), and once formed, may not be reversed with improved glycemic control. Potential scavenger mechanisms exist, however, by which AGEs could be removed from biological systems (11). If these degradative pathways are active and the production rates of AGEs can be reduced, it is possible that more favorable glycemic control could reduce the formation or increase the degradation of AGEs and result in lower tissue levels of these toxic products. This study is designed to evaluate the relationship be-

tween glycemic control over a 3-yr period and tissue levels of AGEs among a carefully characterized population of individuals with type I diabetes.

RESEARCH DESIGN AND METHODS— Eligible subjects with type I diabetes, as defined by the National Diabetes Data Group (12), were selected sequentially from the group of patients regularly attending the Diabetes Clinic at the Dartmouth-Hitchcock Medical Center. The study, which was approved by the human studies committee, was explained, and informed consent was obtained.

To be eligible to participate, patients must be 20–54 yr of age, have diagnosed type I diabetes, have normal renal function based on a serum creatinine within the normal range (0.6–1.5 mg/dl), and be willing to undergo a punch biopsy of the skin.

We entered 50 willing and eligible subjects into the study. We were unable to obtain an adequate skin biopsy on 2 of these subjects, leaving a total of 48 subjects.

Determination of diabetic sequelae

We conducted a complete history and physical examination and evaluated each subject for specific diabetes-related sequelae.

A single retinal specialist (Gault Farrell, MD) examined each subject to determine the degree of retinopathy. The specialist classified each subject's degree of retinopathy as none, background, preproliferative, or proliferative. To determine renal function, we collected two 24-h urine specimens to determine total protein, albumin, and creatinine clearance. Albumin was determined by RIA with a double antibody method (Diagnostic, Los Angeles, CA), and total protein and creatinine clearance was determined by the clinical lab at the Dartmouth-Hitchcock Medical Center. Based on 24-h urinary albumin excretion, subjects were classified as having normal renal function (≤15 mg/24 h), being microalbuminuric (16–150 mg/24 h), or having gross proteinuria (>150 mg/24 h) (13). Mean creatinine clearances for the three groups were 2.01, 1.87, and 1.58 ml/s or 118, 110, and 93 ml/min respectively.

Determination of AGEs in skin collagen

To determine the level of AGEs in collagen, a modification of the method of Monnier was used (6). Skin samples were obtained from the upper right buttock by a 4-mm punch biopsy, and within 30 min, they were snap frozen in liquid nitrogen. The epidermis was removed by sharp dissection under the dissecting microscope, and after mincing, 4–15 mg of the tissue was further disrupted with a Polytron tissue homogenizer for 60 s. The tissue was delipidated with chloroform-methanol (2:1) for 12 h, washed, and suspended in 0.02 M HEPES buffer (pH = 7.5) containing 0.1 M calcium chloride. The pellet was digested in the same buffer containing 280 U of type VII collagenase (Sigma, St. Louis, MO) for 48–72 h at 37°C with constant shaking. We compared the adequacy of the digestion in subjects with short or long duration diabetes and in those with and without vascular sequelae and found that the pellet remaining after digestion contained <3% of the total tissue collagen in all subjects. To assay for collagen-linked fluorescence, samples were read in a spectrofluorimeter (model LS-3B Fluorescence Spectrometer, Perkin-Elmer/Cetus, Norwalk, CT) at excitation/emission readings of 370/440 and 335/385 nm. Fluorescent maxima were found at these two levels when collagenase digests of skin samples were scanned. All readings were corrected for an enzyme blank, and hydroxyproline content was determined by the method of Stegemann and Stadler (14) and converted to collagen assuming a hydroxyproline content of 14%. Fluorescence data are expressed as arbitrary FU/mg of collagen. The within-assay CV for collagen-linked fluorescence was 6.2%, and the between-assay CV was 12% for autopsy control skin samples from the same subject assayed with each run.

Determination of HbA_1

Total HbA_1 was determined by the clinical laboratory at the Dartmouth-Hitchcock Medical Center with ion exchange mini columns (Isolab, Akron, OH). The labile Schiff base was removed before performing the assay by a 60-min exposure to 4% boric acid solution by using Isolab's labile fraction eliminator. The range of normal for this assay is 5.5–8.0%. A mean of 7.1 ± 3.4 HbA_1 values per subject were available from the preceding 3 yr. HbA_1 determinations were performed with a mean frequency of every 4.3 mo for the entire study population.

Statistical analysis

The goal of this analysis is to examine the relationship between glycemic control over a 3-yr period and tissue levels of AGEs at the end of that period. We examined glycemic control, based on HbA_1 determinations, during three separate time intervals: *1*) past 3 yr, 2) past 1 yr, and 3) current. To determine 3-yr glycemic control, we used the mean of all HbA_1 measurements over the preceding 3 yr. Those subjects (primarily those with short-duration diabetes) without HbA_1 measurements in each of the 3 yr before the biopsy were excluded from the analysis of 3-yr control and AGEs. To determine 1-yr glycemic control, we used the mean of all HbA_1 measurements in the year before the skin biopsy. Finally, for current glycemic control, we used the single HbA_1 measurement taken closest in time to the skin biopsy. In 75% of cases, these measurements were taken on the same day, whereas in 92% of the cases, the measurements were made within 3 wk, with the remaining 8% performed within 8 wk. Subjects without an HbA_1 measurement within 8 wk of the biopsy were excluded from the analysis

Table 1—*Clinical characteristics of study subjects*

n	48
AGE (YR)	36.1 ± 8.4
DURATION OF DIABETES (YR)	19.4 ± 8.5
PERCENTAGE MALE (%)	64
PROPORTION OF TOTAL WITH RETINOPATHY (%)*	38
PROPORTION OF TOTAL WITH NEPHROPATHY (%)†	48

Data are means ± SD.
*Preproliferative or proliferative neuropathy.
†Microalbuminuria or gross proteinuria nephropathy.

of current glycemic control and tissue glycosylation.

We examined glycemic control first as a continuous variable and then as a categorical variable. Specifically, we used Pearson's correlation coefficients to examine the correlation between glycemic control during the study period and tissue levels of AGEs at the end of the interval. Correlations were examined at each of the excitation/emission readings (370/440 and 335/385 nm). Because the tissue fluorescence levels are slightly nonnormally distributed, we also analyzed the data by using log transformations of the data.

For the categorical analysis, we used the mean HbA_1 levels during each time period to classify the subjects into one of three categories of glycemic control: *1*) good control (mean HbA_1 ≤8.5%), 2) fair control (mean HbA_1 >8.5 and ≤10%), and 3) poor control (mean HbA_1 >10%). Thus, each subject with available HbA_1 data was classified in terms of glycemic control for each time interval (i.e., past 3 yr, past 1 yr, and current).

We used a standard one-way ANOVA to compare the mean levels of AGEs among those with good, fair, or poor glycemic control. To adjust for the possible confounding effects of age, sex, and duration of diabetes, we used an ANCOVA to determine adjusted mean AGE levels for each category of glycemic control.

We also analyzed the level of correlation between the degree of diabetic nephropathy (normoalbuminuric, microalbuminuric, and grossly proteinuric) or retinopathy (none, background, preproliferative, or proliferative) and tissue levels of AGEs by using Spearman's correlation coefficients.

RESULTS — The clinical characteristics of the 48 subjects included in this analysis can be found in Table 1. Their mean age was 36.1 yr (range, 21–53 yr), and the average duration of their diabetes was 19.4 yr (range, 4–34 yr). Men accounted for 64% of the subjects. At entry into the study, 38% had preproliferative or proliferative retinopathy and 48% had some degree of diabetic nephropathy (microalbuminuria or gross proteinuria).

We examined the correlation between HbA_1 levels and AGEs at each of the two fluorescent wavelengths by using Pearson's correlation coefficients. The results of these analyses can be found in Table 2. A statistically significant correlation was observed between glycemic control in the past 1 and 3 yr and tissue levels of fluorescent products, with stronger correlations found for AGEs measured at the 335/385 nm wavelength. The correlation was weaker between current glycemic control and AGEs. In this analysis, we noted that the distribution of tissue levels of fluorescent products was slightly nonnormal, so we also examined the correlation after log transforming the AGE levels. This analysis showed slightly stronger correlations. For example, the correlation between control in the past 1 yr and tissue levels of fluorescent 370/440 nm products was 0.30 ($P = 0.04$). The same analysis using log-transformed fluorescent products yielded a correlation of 0.34 ($P = 0.01$). The log transformations yielded a similar increase in the correlation coefficients for all analyses.

Table 2—*Correlation analyses of glycemic control and AGEs*

	FLUORESCENT WAVELENGTHS	
	335/385 NM	370/440 NM
GLYCEMIC CONTROL		
CURRENT (*r*)	0.20	0.20
P VALUE	0.170	0.175
PAST 1 YR (*r*)	0.33	0.30
P VALUE	0.022	0.041
PAST 3 YR (*r*)	0.40	0.30
P VALUE	0.008	0.058

The mean levels of AGEs for those with good, fair, or poor glycemic control during each time period can be seen in Table 3. We have presented the mean levels of AGEs separately for the two fluorescent wavelengths of interest. We used an ANOVA procedure to test the null hypothesis that no difference occurs among the mean AGE levels during each time period for those with good, fair, and poor control. The *P* values associated with the overall *F* test from the ANOVA can be seen in Table 3. We consistently found that the mean levels of fluorescent products was lowest among those with good glycemic control and highest among those with poor glycemic control during each time period. These differences were statistically significant when examining glycemic control over 3 yr for both wavelengths, of borderline significance for the past 1 yr of glycemic control, and did not achieve statistical significance when current glycemic control was examined in relation to levels of fluorescent products. The results from the ANOVA were similar at the two fluorescent wavelengths examined, but the differences in mean levels

Table 3—*Mean levels of AGEs by glycemic control category*

	n	Fluorescent wavelengths (FU/mg collagen) 335/385 nm	370/440 nm
Glycemic control			
Current	46		
Good	19	13.6 ± 4.4	10.0 ± 4.2
Fair	16	14.7 ± 4.9	11.1 ± 4.2
Poor	11	15.5 ± 3.4	11.6 ± 3.2
P value		0.52	0.50
Past 1 yr	48		
Good	15	12.5 ± 3.7	9.0 ± 3.8
Fair	22	14.9 ± 4.9	11.2 ± 4.2
Poor	11	16.8 ± 3.0	12.6 ± 2.8
P value		0.05	0.06
Past 3 yr	42		
Good	13	11.4 ± 3.3	8.1 ± 3.8
Fair	21	14.9 ± 4.1	11.2 ± 3.5
Poor	8	16.8 ± 2.0	12.5 ± 2.0
P value		0.003	0.011

Data are means ± SD.

were somewhat smaller at fluorescent 370/440 nm wavelength.

In Table 4, we present the results of the ANCOVA used to control potential confounding by age, sex, and duration of diabetes. The adjusted mean levels for those with good, fair, or poor glycemic control did not differ substantially from the unadjusted means presented in Table 3. Again, the mean AGE levels are lowest for those with good glycemic control and highest for those with the poorest control. The use of log transformations for the AGE data did not substantially alter the results (although the strength of the associations were in general slightly stronger), so we have again elected to present these data by using nontransformed fluorescent products data (Tables 3 and 4).

When the relationship between the degree of diabetic nephropathy and retinopathy and tissue levels of AGEs was assessed with Spearman's correlation coefficients, highly significant correlations were found. The correlation coefficients for the relationship between nephropathy and AGEs (measured by fluorescence at 335/385 and 370/440 nm) were 0.59 and 0.49 with *P* values of 0.0001 and 0.0005, whereas those for the degree of retinopathy and AGEs were 0.40 and 0.27 with *P* values of 0.005 and 0.06, respectively.

Finally, because diabetic nephropathy may be related to glycemic control and also to tissue levels of AGEs, we were concerned that the observed relationship between glycemic control and AGEs might be confounded by existing diabetic complications. To explore this possibility, we excluded all subjects with any degree of nephropathy (i.e., microalbuminuria or gross proteinuria) and reexamined the relationship between glycemic control and AGE levels for only those subjects with no evidence of nephropathy ($n = 25$). Although the numbers of subjects is smaller (52% of the entire study population), the results are very similar to the earlier results for the entire group. For example, for 3-yr glycemic control, tissue AGE levels at 335/385 nm for those with good, fair, and poor control were 10.5, 13.0, and 16.5, respectively, and the *P* value for differences across categories was 0.02.

CONCLUSIONS— These data demonstrate that tissue levels of AGEs differ significantly across levels of glycemic control when a population with type I diabetes is examined over extended time periods. Those with the most favorable glycemic control had the lowest AGE levels, and those with the poorest control had the highest AGE levels. Although age and duration of diabetes have been shown previously to be associated with significant increases in tissue levels of AGEs (6,7), these analyses demonstrate that the relationship between glycemic control and AGEs is independent of age and duration of diabetes as well as sex, because the adjusted levels of AGEs relating to different categories of glycemic control do not differ from the unadjusted levels. Furthermore, note that the observed relationship between glycemic control and AGEs in these data was similar in individuals with and without diabetic vascular sequelae. This supports a relationship between glycemic control and tissue levels of AGEs that is independent of potential confounding by the presence of microvascular sequelae.

The relationship between the level of AGEs in collagen and glycemic control over the preceding 1 and 3 yr in subjects with type I diabetes, suggests that glycemic control may be predictive of the balance between formation and degradation of these long-lived AGEs in tissues. In the only other study of tissue glycosylation and glycemic control that we are aware of, Lyons et al. (8) have shown improvement in early glycosylation (Amadori) products in subjects with type I diabetes after 4 mo of tight glycemic control, but they were not able to demonstrate changes in AGEs or oxidation products over this time. In this study, the level of glycemic control over a period as short as 1 yr was predictive of AGE levels, which could indicate that the tissue content of these chemically irreversible products can be modified over a

Table 4—*Adjusted levels of AGEs by glycemic control category*

	Fluorescent maxima wavelengths (FU/mg collagen)	
	335/385 nm	370/440 nm
Glycemic control		
Current		
Good	14.3 ± 4.1	10.4 ± 4.0
Fair	14.2 ± 4.1	10.7 ± 3.9
Poor	15.1 ± 4.1	11.4 ± 3.9
P value	0.449	0.470
Past 1 yr		
Good	13.5 ± 4.1	9.7 ± 3.9
Fair	14.4 ± 3.9	10.8 ± 3.8
Poor	16.5 ± 4.0	12.5 ± 3.8
P value	0.027	0.050
Past 3 yr		
Good	12.8 ± 3.6	9.0 ± 3.6
Fair	14.4 ± 3.4	10.8 ± 3.4
Poor	16.0 ± 3.4	12.0 ± 3.4
P value	0.001	0.008

Data are means ± SD. All mean levels were adjusted for age, sex, and duration of diabetes by using ANCOVA.

time period shorter than that generally considered necessary for the development of diabetic vascular complications (15). Although we did not directly measure the turnover of AGE modified collagen, our results also suggest the possibility that degradative pathways may be capable of modifying tissue levels of AGEs if the rate of formation is reduced by lowering mean levels of reactive sugars. Potential mechanisms, particularly those mediated by macrophages (11), which could lead to the degradation of AGE-containing proteins have been identified. These pathways could continue to function normally in the face of decreased formation of AGEs secondary to improved ambient glycemia and could lead to a reduction in tissue levels of AGEs. It is also possible that improved glycemic control could lead to a direct increase in the activity of these degradative pathways, resulting in reduced AGE levels. Because these investigations measure AGE accumulation and presumably reflect the rate of formation, a prospective study with alternating periods of poor and good glycemic control is needed to fully assess the relative rates of formation and degradation of AGEs.

We did not find that a single HbA_1 determination taken in close proximity to the skin biopsy was predictive of tissue levels of AGEs. This suggests that a single measurement of short-term glycemic control (1–2 mo) is not an accurate predictor of the tissue levels of AGEs in individual subjects and indirectly suggests that a single HbA_1 is not predictive of longer term glycemic control. However, the statistically significant associations between glycemic control over longer time periods (1–3 yr) and AGE levels could have resulted in part from the more stable estimate of the individual's usual glycemic control resulting from the use of the average of multiple measurements during those time periods.

Studies by Monnier (6,7) have demonstrated a relationship between AGEs and diabetic vascular sequelae, and recent data from our laboratory have confirmed and extended this association. Indeed, an extensive body of information has accumulated that supports an important role for AGEs in the pathogenesis of diabetic sequelae (1). Because our findings suggest that glycemic control may predict AGE levels and previous studies have shown a relationship between AGEs and the presence of diabetic sequelae (6,7), our results indirectly suggest a relationship could exist between glycemic control and long-term diabetic sequelae. We also have observed a direct association between glycemic control and diabetic retinopathy and nephropathy in this study, although these findings are limited by the cross-sectional nature of the analyses. Note that collagen in vascular structures is presumably the site of pathology in diabetes, and although skin collagen is similar to other collagens in its slow turnover, it is possible that tissue remodeling and receptor-mediated turnover processes may vary in different collagen-containing tissues (16).

The relationship between glycemic control and tissue levels of AGEs appears to be stronger for fluorescent products measured at 335/385 nm than for those measured at 370/440 nm, which suggests that glycemic control may correlate most closely with specific fluorescent AGEs such as pentosidine, which has similar excitation and emission characteristics (5). Although the method used to measure tissue levels of AGEs in this study (i.e., fluorescent products measured at 335/385 and 370/440 nm) detects families of AGEs rather than specific products, the measurement of AGEs by this method correlates well with the measurements of the specific cross-link, pentosidine (17), and has generally represented the standard method for the quantification of these products. It also is not clear at this time which of the many possible AGEs formed at increased rates in diabetes and aging play the most important role in the development of vascular sequelae. Because several new assays for AGEs have been reported recently (18–20) and others are under development, further studies to

investigate the relationship between specific AGEs quantified with these assays and glycemic control in well-characterized clinical populations are needed to confirm our findings, as well as to further assess the role of AGEs in the development of diabetic sequelae.

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